Risk by Human Health for Invasion of *Pteridium arachnoideum*, in Bolívar, Ecuador Ptaquiloside ´S Content in Fronds and in Milk

Ángela Calderón Tobar

Augusta Jiménez Sánchez

State University of Bolívar
Av.Ernesto Che Guevara s/n y Av
Gabriel Secaira
Guaranda, Bolívar, Ecuador

Luz María Sánchez Perera

Betty Mancebo Dorvigny

Evangelina Marrero Faz

Department of Chemistry
Pharmacology and Toxicology
National Center of Animal Health
Mayabeque, Cuba

Abstract

Pteridium spp. is a very invasive plant in the world. It has a carcinogen toxic substance named ptaquiloside. Recently were make a first report of presence the Enzootic Hematuria Bovine (EHB) in San Miguel, Bolivar province, Ecuador. This report describes the presence of high invasion by Pteridium arachnoideum (bracken fern) in areas of cattle grazing. For also, the present work had as objective study the level of ptaquiloside in fronds of bracken fern in different farms considering different altitude. It was make determination of ptaquiloside residual level in milk from cantons with high invasion of bracken fern. In both case were use High Pressure Liquid Chromatography (HPLC) by extern standard using pterosin B, as stable product of ptaquiloside. The study report high concentration of ptaquiloside in fronds of bracken fern no dependent of altitude. High level of residual toxic compounds were find milk sample. High level of toxic in milk constitute a potential risk by human health in Ecuador.

Keywords: Pteridium arachnoideum, ptaquiloside, bracken fern, milk, HPLC

Introduction

Bracken fern (Genus *Pteridium*) is a complex genus whose taxonomy is in a state of current revision. Bracken fern (*Pteridium aquilinum* (L.) Kuhn) is one of the most common plant species on Earth. The fern is found on all continents except from Antarctica where it occurs as a common weed on agricultural lands, as part of the primary bush or below canopy openings inside forests. Bracken contains a wide range of secondary metabolites of which some are toxic towards humans and other living organisms. The most prominent of these compounds is the norse squiterpene glucoside ptaquiloside Figure 1. [1].

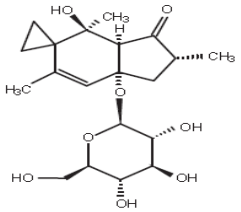

Figure 1.Chemical Structure of Ptaquiloside, the Major Carcinogenic Constituent of Bracken Fern (*Pteridium aquilinum*)

Ptaquiloside causes a wide range of diseases in animals ranging from thiamine deficiency to cancer. Especially bovine urinary bladder cancer known as Bovine Enzootic Haematuria occurs widespread in some parts of the world. Ptaquiloside is also suspect of causing human gastric and esophageal cancer, as the carcinogen readily passes into bovine milk of cows browsing on Bracken and may leach from Bracken stands into drinking water supplies. The consumption of the plant is common in Japan, though pre-treatment with boiling water or soda ash reduces the carcinogenicity. The risk of esophageal cancer is increasing approximately by 2.1 in men and 3.7 in women [2]. Ptaquiloside has been identify in the milk produced by bracken-fed cows [3;4]; the concentration in milk has been found to be about 8.5% of the amount ingested by the cow and is linearly dependent on the dose. The authors indicate that, in their view, it is 'certainly likely' that ptaquiloside in milk is responsible for the connection between bracken infestation and the incidence of gastric cancer in populations of farmers in habiting cattle-range areas in Costa Rica and, other countries where bracken growth is dense [5].It is strongly suggesting the need to avoid the distribution of this into the food chain. In deed neoplasia has been caused by feeding rats with the milk of cows or rats fed on a bracken fern diet [6; 7]. A possible spatial association between bracken and cancer in England and Wales has been examined [8]. While aerial exposure to spores may be a problem given the development of neoplasia in mice treated with spores [9;10] and the observation that this treatment leads to DNA adducts in upper gastrointestinal tissues[11;12].

Ptaquiloside is not toxic *per se*, as it has to be activated (i.e., transformed to the unstable diedone, which is believed to be the ultimate carcinogen). Activation takes place at alkaline conditions, which help explain the localization of tumors in bovine (pH(saliva): 8.1 – 8.2; pH(urine): 7.5 - 8.5). It is the electrophilic properties of the cyclopropane ring system that cause the alkylating properties of activated ptaquiloside (the dienone). The dienone reacts willingly with nucleophiles such as water, alcohols, amines, thiol and sulphide groups in amino acids, DNA etc.[13] (Figure 2).

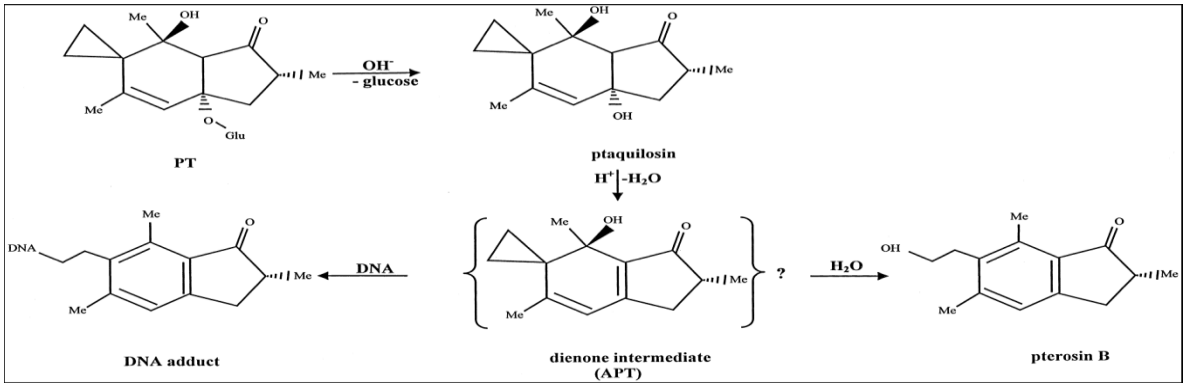

Figure 2: Propose Scheme of Ptaquiloside Reaction Pathway

In 2011 was reported the presence of Bovine Enzootic Haematuria (BEH) in Ecuador, with certain clinical and related environmental conditions coincide with regions of South America and this studied shown the unique variety of *Pteridium* identified was *Pteridium arachnoideum* [14]. Considered the high invasion for this specie and the risk for animals and human health, the objective of the present work was determinate the levels of Ptaquiloside by HPLC in young and adult fronds fern at different altitude and in milk samples from three regions of Bolivar province, Ecuador with presence of BEH.

Materials and Methods

Experimental Design

The study was carried out as three separate cantons from Bolivar province, as follows: San Miguel (Sandalan, Quebracha, Regulo de Mora, Changuil Medio, Mata Palo), center of Bolivar province, altitude 800 to 2469 Mons and temperature of 22 -08°C. The second was Chimbo (Tronador Grande), altitude 800 to 2500 Mons and temperature of 22 - 8°C. Echeandía (San Pablo de Echeandía) was the third canton; it was altitude 300 to 1400 Mons and medium temperature of 20°C. We showed the map of Bolívar and their cantons. (Figure.3).

Figure 3.Map of Bolívar Province- Ecuador and Their Cantons

In each region were made a visual determination of distribution and contamination of grasses with *Pteridium* sp. at different altitudes: low, medium and high. We collected samples in each zone and altitude from young fronds (fronds with less than 30 cm of high) and adult fronds (fronds with more than 1 meter of high). One sample was collected from San Miguel at 2114 mons with young and adult fronds by obtain pterosin B as reference in analytical techniques. Table 1 showed the regions test for the presence studies.

Table 1: Bracken Fern Collection Samples of in Different Cantons of Bolívar Province, Ecuador

Place or canton	Alti-tud (Mons)	Collection Date	Collection hour	Frond high	Bracken fern Dominance	Climate	Num-berof farms.
Chimbo	1635	30/06/2011	12:30 PM	Less than 30 cm	Abundant ++	Sub-tropical humid. Media Temp: 16°C	15
Chimbo	1635	30/06/2011	12:30 PM	More than 1 m	Abundant ++		
Chimbo	1599	30/06/2011	12:50 PM	Less than 30 m	Abundant ++		
Chimbo	1599	30/06/2011	12:50 PM	More than1 m	Abundant ++		
Chimbo	1404	30/06/2011	13:20 pm	Less than 30 cm	Abundant ++		
Chimbo	1404	30/06/2011	13:20 pm	More than 1 m	Abundant ++		
Echeandía	1354	01/07/2011	11:00 PM	Less than 30 cm	Abundant +	Sub-tropical humid. Media Temp: 20°C	10
Echeandía	1354	01/07/2011	11:00 PM	More than 1 m	Abundant +		
Echeandía	1237	01/07/2011	11:15 PM	Less than 30 cm	Abundant +		
Echeandía	1237	01/07/2011	11:15 PM	More than 1 m	Abundant +		
Echeandía	798	01/07/2011	11:30 PM	Less than 30 cm	Abundant +		
Echeandía	798	01/07/2011	11:30 PM	More than 1 m	Abundant +		
San Miguel	2114	02/07/2011	10:00 AM	Less than 30 cm	Abundant +++	Sub-tropical humid to cold. Media Temp: 22-08°C	32
San Miguel	2114	02/07/2011	10:00 AM	More than 1 m	Abundant +++		
San Miguel	1691	02/07/2011	10:30 AM	Less than 30 cm	Abundant +++		
San Miguel	1691	02/07/2011	10:30 AM	More than 1 m	Abundant +++		
San Miguel	1389	02/07/2011	11:00 AM	Less than 30 cm	Abundant +++		
San Miguel	1389	02/07/2011	11:00 AM	More than 1 m	Abundant +++		

Samples of milk were collected from different farmers of zones in studies. Five samples of milk (100 ml) were collected by each producer. Milk produced by cows with Bovine Enzootic Haematuria was mark.

Isolation and Purification of Pterosin B, as Reference

Fresh fronds of *Pteridium arachnoideum* were curt in peace of 5 - 10 mm with a court scissor. A sample of 500 g was extracted with 1 L of distilled water at ambient temperature (37°C) with agitation by 1 hour and after it was extracted in ultrasonic bath for 45 minutes. It was filter. Residue was discarding and supernatant was extracted twice with 100 ml of petroleum ether. Ether was discarding. Aqueous extract was alkalinized with 1 M Sodium Hydroxide to pH >11. It was hot at 40°C for 1 h. After, it extract was acidify with hydrochloride acid 5 m to pH <2. It was resting 10 minutes and after extracted three times with 100 ml of dichloromethane. The organic layers was collected and dried at reduce pression (Diagram 1).

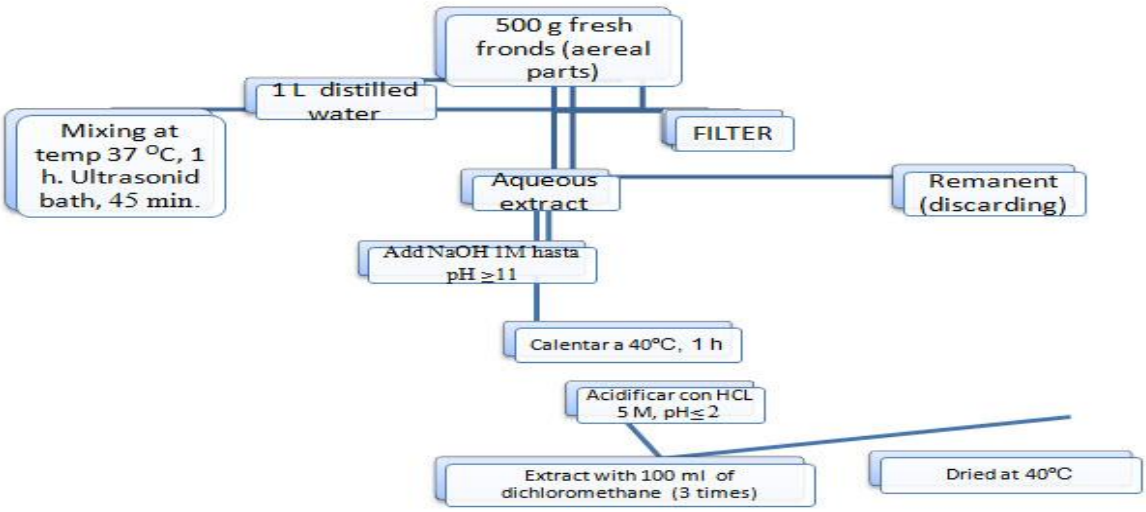

Diagram 1: Extraction of Pterosin B from *Pteridium arachnoideum*

Crude of pterosin B was proceeding to purification with vacuum column chromatography. Crude was dissolved in methanol: dichloromethane 1:1 and it was absorbed in 1 g of silicagel 60 by column chromatography (Kieselgel 60, 0.015 – 0.040 mmm, Merck). It was put on column with 20 g of silicagel 60 F, kieselgel 60 F, Merck. The column was eluted with hexane (100 ml); hexane: ethyl acetate 90:10 (300 ml); hexane: ethyl acetate 70:30 (200 ml); hexane: ethyl acetate 50:50 (200 ml); hexane: ethyl acetate 30:70 (100 ml) and end with ethyl acetate (200ml). Pterosin B was obtained in hexane: ethyl acetate 70:30 fraction. It was verified by Thin Layer Chromatography, silica gel 60 F254, merck, Germany with elution ethyl acetate: dichloromethane 40:60 and detection at 254 nm observing a at Rf = 0.64.

Ptaquiloside ´s Determination by HPLC, from samples of *Pteridium arachnoideum*

Samples from *Pteridium arachnoideum* were processed according to the method of Agnew and Lauren[15] with some modifications in the following diagram (Diagram 2).

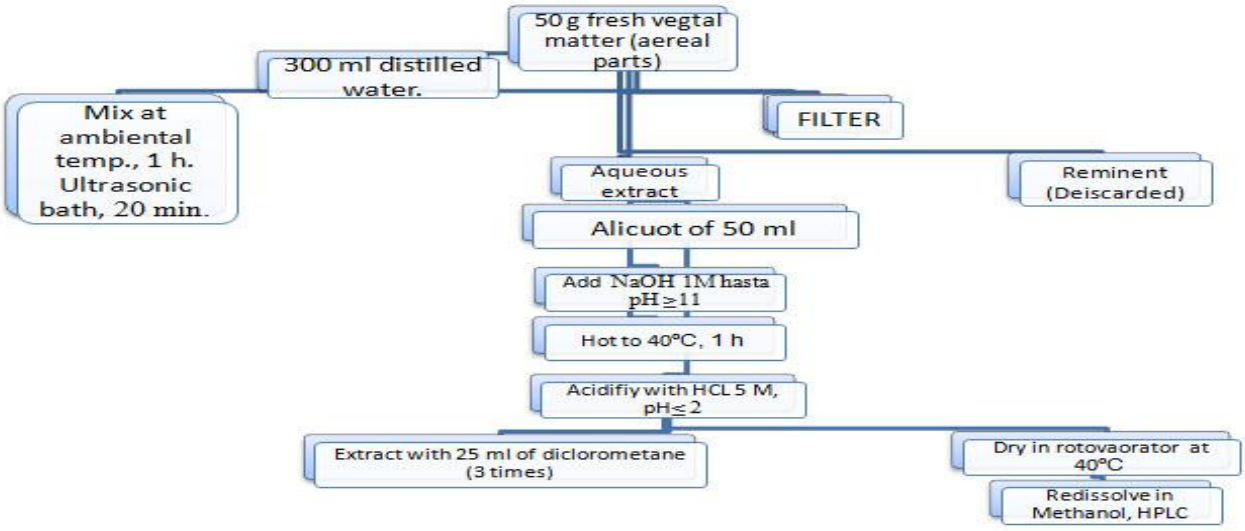

Diagram 2: Extraction and Determination of Ptaquiloside through Conversion at Pterosin B from Samples of *Pteridium arachnoideum* by HPLC

The dried samples were redis solved in 1 ml of Methanol; they were put in vials by auto sampling HPLC. Chromatography conditions by HPLC were: Equipment HPLC: ULTIMATE 3000 (DIONEX), Column Dionex RP 18, 12.5 x 0.4 cm i.d., 40 µ, Ambient temperature; isocratic with Methanol: Agua 65:35 as elution system, injector 20 µl; Flux = 1 ml/min, Detection Variable Ultraviolet at λ= 260 nm, with a program software CROMELEON.

Patron curve were running with Pterosin B as external patron in the concentrations 1; 0.9; 0.7; 0.5; 0.1; 0.07; 0.05 y 0.01μg/ml. It correspond one curve by the determination of concentration in the samples and other to determinate detection and quantification limits by the technique.

Recover, Detection and Quantification Limits Determination

Recover was analysis on samples with a known quantity of pterosin B. Following the extraction and determination similar manner and determinate the percentage of recuperation of this parameter following the formula:

$$\text{Waiting concentration} = \frac{\text{Sample quantity + Added quantity}}{\text{Sample volume + added volume}}$$

$$\% \text{ recuperation} = \frac{\text{Observed concentration}}{\text{Wait concentration}} \times 100$$

Sensibility (Detection and Quantification Limits)

Sensibility were determinate by the method describe by Quatrochi *et al*, [16]. It were made a calibration curve for the standard Pterosin B in the range normal by the sample, between 1 – 0,1 mg/ml and it was determinate the pendent (m). After it was made other calibration curve in low concentration of standard and each point was injecting tree times in HPLC. It was determinate a new equation of the curve (A= mx Conc. + b) ant it extrapolate the answer by cero concentration (Area = b) obtain an answer estimate for blank Ybl. It was determinate the standard deviation (S) by each point in the curve and it calculate the recta, equation S= m(A) + b, and it extrapolate.S at cero concentration obtaining Sbl estimate corresponding at standard deviation of the blank.
DL and QL is calculated by:

$$LD = \frac{Y_{bl} + 3S_{bl}}{m} \; 1/\sqrt{n'}$$

$$LQ = \frac{Y_{bl} + 10S_{bl}}{m} \; 1/\sqrt{n'}$$

Determination of dry weight and humid percentage in sample of fronds: One gram of each sample was proceeding by humid percentage and dried weight by IR balance.

Level from Ptaquiloside ´s Determination by HPLC, from Milk Samples

Milk samples were collected (100 mL) in different cattle farm in each canton in study, with presence of *P. arachnoideum* invasion in grazing. Milk´s samples were collect proceeding of cows with and within clinical signs evident of BEH. It was sampled 15 % of total cattle in each farm (n= 84). Samples were hope in amber glass bottle, once by cow and it was identify itself. Samples proceeding of first manual milking in the morning. It were conserve in cool condition (within pasteurize), after it were worked in the Agricultural Sciences Faculty Lab, Bolivar State University (Guaranda) and Phytochemical lab in Chemical Science Faculty, Ecuador Central University (Quito). Fields sample were at 2210 – 778 Mons.

HPLC Methodology by Ptaquiloside level Determination in Milk Samples

Samples were process by Alonso- Amelot *et al* (1993) with some modification. Twenty- five aliquot each fresh milk sample were treat with 40 mL of methanol with agitation at ambient temperature (37°C) by 30 minutes, by proteins precipitation. After, it were filter and centrifuge at 3000 rpm by 15 minute. Supernatant was treat with 25 mL of acetonitrile by lipid precipitation. It was centrifuge discard pellet (lipids and proteins) and after it were add 1 g of sodium chloride at supernatant. Extraction with dichloromethane made by fat elimination. Aqueous phase were treat with sodium hydroxide (0.003 N) to basic pH ≥11, by conversion at pterosin B. It was mix at 36°C during 2 hours. Second extraction made with dichloromethane (3 times x 15 mL) and after organic extract were dry at reduce pressure at 40°C. It were dissolve in 1 mL of methanol HPLC quality and were hope in vials by HPLC (Diagram 3).

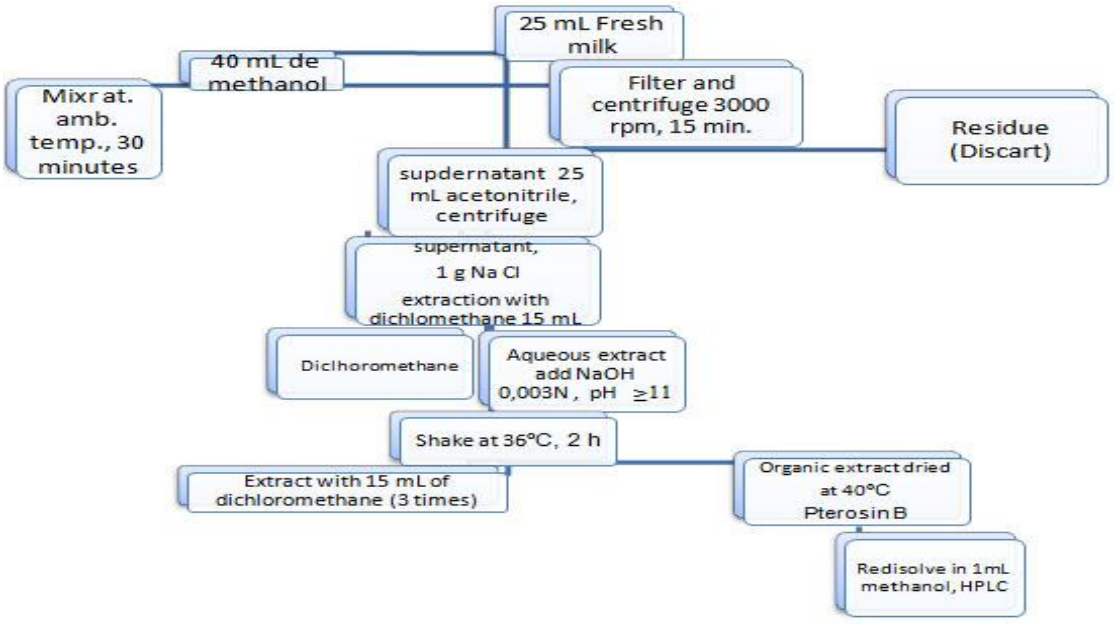

Diagram 3.Extraction and Determination of Ptaquiloside through Conversion at Pterosin B in Fresh Milk Samples by HPLC

Chromatography conditions by HPLC were: Equipment HPLC: ULTIMATE 3000 (DIONEX), Column Dionex RP 18, 12.5 x 0.4 cm i.d., 40 µ, Ambient temperature; isocratic with Methanol: Agua 65:35 as elution system, injector 20 ul; Flux = 1 ml/min, Detection Variable Ultraviolet at λ= 260 nm, with a program software CROMELEON.

Patron curve were running with Pterosin B as external patron in the concentrations 1; 0.9; 0.7; 0.5; 0.1; 0.07; 0.05 y 0.01 µg/ml. It correspond one curve by the determination of concentration in the samples and other to determinate detection and quantification limits by the technique by Quatrocchi *et al*. 1992 (16).

Recover, Detection and Quantification Limits Determination

It was used one milk sample within contamination (negative control) by specificity/selectivity determination. It was added pterosin B (2 mg). Three replicate by it study. Extraction and quantification processed similar to previously and it was determinate recover percentage.

Statically Analysis

The results were analyzed by comparison of means by Student's tand correlation with $p < 0.05$ using the statistical programEPIDAT3.1.

Results and Discussion

Pterosin B, stable form of toxic compounds ptaquiloside, as external patron with 90 % of pure estimated by Thin Layer Chromatography (TLC) and by HPLC with reference substance was obtain (Figure 4).

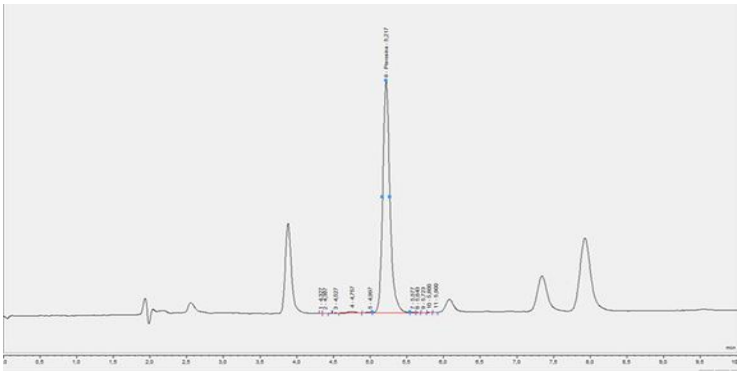

Figure 4: Chromatography Profile of Pterosin B Patron by HPLC

Figure 4 shown chromatography profile of Pterosin B at tr = 5,217 minutes but it has some impure in low concentration.

This result confirms reports of other authors who had obtained pure range similar using extraction and chromatography proceeding similar of employed in the present work (13; 1). The indirect Ptaquiloside determination by it stable metabolite Pterosin B is possible by the estequiometric character of the conversion, so this conversion results in an approximate doubling of the UV response and will hence lower the limit of detection, overcoming problems associated with Ptaquiloside handling (17).

In the determination of ptaquiloside using pterosin B in young and adult's fronds of *Pteridium arachnoideum* at three altitude level from San Miguel, Chimbo and Echeandía cantons from Bolivar province shown a detection limit of 1.95 µg/g and quantification limit of 2.47 µg/g, showing a good method sensibility and the recover was 95.5 % (Figure 5).

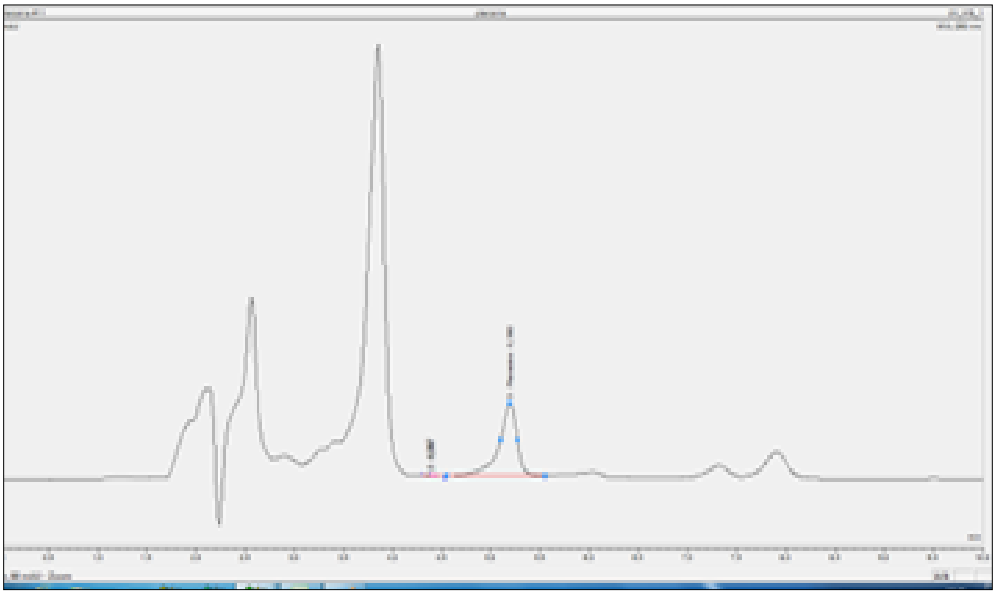

Figura 5: Chromatography Profile of one Sample from *Pteridium arachnoideum*

Detection and quantification limits obtained in the determination of Pta in fronds of *Pteridium arachnoideum* were superior at reports by Agnew y Lauren 1991 (18). Rasmussen *et al*. 2008 (1) who had found detection limit of 5 µg/g. the best results obtain in present study will be associate to the best analytical capacity employed in HPLC technology and chromatography conditions. Its factors increase the resolution and sensibility of the method. Recover of 95.5 % will be in relationship at the application of modification in the extraction including ultrasonic bath, with best contact of solvent- superficies in the matrix helping the extraction process in the analysis of toxic principle (19).

Figure 6, shown the results that evidence not corelations between altitudes in the different cantons studied and the concentration of Pta (µg/g fresh and dried fronds) nor correlation exist between age of the fronds (young and adult fronds) and concentration of Pta. For also, the concentration of Pta at any altitude (798 a 2114 m asl) were similar in the three cantons with general mean altitude of 1469 m asl and mean concentration of Pta of 573,38 ug/g of fresh fronds young and adults and 1983,97 µg/g of dried fronds young and adults. However, in the Chimbo canton exist the major means concentration of Pta. So in fresh state of the young and adult fronds (659, 97 µg/g) and dried state of young and adults fronds (3255,71µg/g) at mean altitude of 1546 asl and the low concentration were in Echeandia canton in fronds adults fresh and dried with 107,7 y 376,57 µg/g respectively at a altitude of 798 m asl.

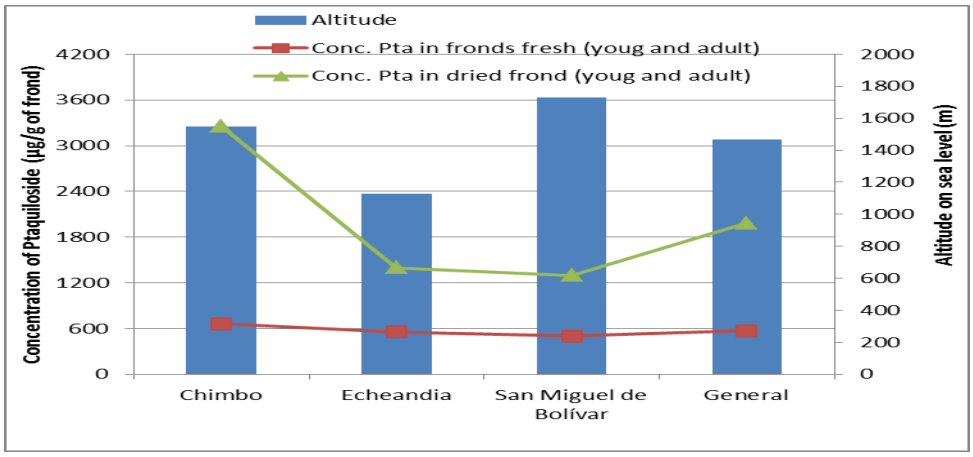

Figure 6: Means Concentration of Ptaquiloside (µg/G of Fresh and Dried Fronds) from Plants Young and Adult of *Pteridium arachnoideum*, at Different Altitude in San Miguel, Chimbo And Echeandía from Bolívar Province, Ecuador. (P= 0.72) and (P= 0.51) (X ± SD)

In Figure 7, shown the mean Ptaquiloside concentration in young fronds (588, 58 µg/g frond) and in adult (558,18 µg/g frond) from *Pteridium arachnoideum. T*here are not significant, equal to mean concentration in dried young frond (2140,01µg/g de frond) and adult (1827,83µg/g frond), however in both case the concentration of Pta had increased significantly in dried state of vegetal matter to rest the high percentage of humid in this plant´s organ. The mean general concentration in young and adult fresh frond was 573,38µg/g an in young and adult dried fronds was 1983,97 µg/g.

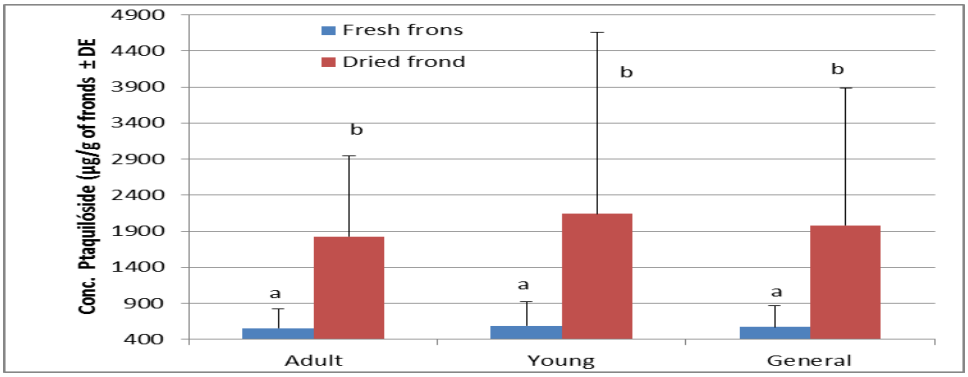

Figure 7: Mean Ptaquiloside Concentration (µg/G of Fresh and Dried Fronds) in Young Plants (< 30 Cm) And Adults (≥ 1 M) Of *Pteridium arachnoideumin* San Miguel, Chimbo and Echeandía Cantons, Bolívar Province. P= 0,001 (X±DE). T De Student

The level of ptaquiloside studies in different species and variety of *Pteridium* from Australia, Sub America and Europe shown correlation between ptaquiloside´s content, stage of growth and geographical variables as altitude (20), latitude (21) and light exposition (22). Somvanshi *et al* (23), found equal correlation at high altitude (500 – 5000 m als) in samples of not bracken (*Onychiumcontiguum)* in India. However, the dates obtain in a present study in the determination of carcinogenic compound in *Pteridium arachnoideum* in Ecuador not shown these correlation respect to increasing of ptaquiloside concentration with altitude and stage of growth of plant in each regions studied, agreeing with our results Rasmussen *et al.* (1), who in a study to examine the variation of Pta in fern sat the farm level, regionally and nationally throughout New Zealand, found no correlation between the content of Pta in the plant and environmental parameters measured on farms region a land national data so that individual samples may tend to support the correlations with latitude and / or altitude, considering these weak trends and that do not have the support of other data performed, so further studies are required to describe potential correlations between environmental parameters. In the chromatogram of fronds of *Pteridium arachnoideum* have another peak at major retention time from Pterosin B with minor height. It could be some isomer or derivate from ptaquiloside. For also, it will be recommendable to use HPLC with Mass Spectrometer or Gas Chromatography/ Mass by it compound identification.

It will be consider that some fern has some pterosin B derives or pterosin relationship. *Pteridium aquilinum* content 20 types of pterosin and pterosides who produce pterosin Z and pterosin A by hydrolysis (24).

In the case of milk sampling of different farms from three canton of Bolivar province affected by EBH using HPLC shown detection limit of 1.95 µg/mL and quantification limit of 2.47 µg/mL. These results shown the method sensibility by determination of ptaquiloside in milk samples analysis. Recover was 89%. Level from ptaquiloside in the samples were correct by dividing by the recovered.

Figure 8shown chromatogram by one milk sample. It shows specificity of method with the presence of major peak correspondence to analysis compound.

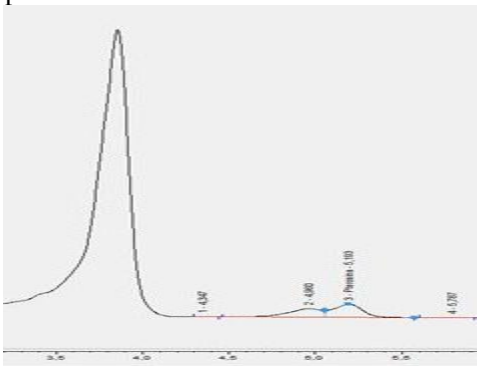

Figure 8: Chromatography Profile form Milk Sample by HPLC

Table 2, maxima concentration of ptaquiloside (µg/mL) in fresh milk founds by HPLC reflex high levels in the three studing cantons with significant difference ($p < 0.0007$). In Chimbo canton has a maxima level in milk of 2641,13 µg/mL in relation with Echeandia and San Miguel who have de 2130,12 µg/mL y 2219,67 µg/mL respectively. It were detected toxic residual in milk of 89,29 % from total samples analysis. Is very important to tell that toxic residual in milk were found in all farms analysis from the three cantons at different altitude so 2210 at 778 als.

Table 2.Ptaquiloside Concentration Levels (µg/Ml) of Fresh Milk Samples from the Cantons Chimbo, San Miguel and Echeandía in Bolívar Province, Ecuador.P< 0, 0007 (X ±DE)

Cantons	Fresh milking samples	Content. Ptaquiloside correge by recover factor 0,89 (µg/mL) in fresh milk. Máximun levels	Mean concentration Ptaquiloside (µg/ml) in fresh milk .	Standard Deviation.	No detected milking samples		% of milk samples with toxic residual.
					n	%	%
Chimbo	**25**	**2641,13**	**1788,26[a]**	**882,60**	**1**	**4**	**96**
Echeandía	**30**	**2130,12**	**797,73[b]**	**671,01**	**3**	**10**	**90**
San Miguel	**29**	**2219,67**	**805, 42[b]**	**640,34**	**5**	**17,24**	**82,76**
General	**84**	**2641,13**	**1130,47**	**852,58**	**9**	**10,71**	**89,29**

(a,b) Mean values with different letter sin super script in the same column indicate statistical differences.
$p<0.05$

Referent at the present found it is associate ptaquiloside elimination in milk from cow intoxicate (25), principally in subclinical form where not appreciate clinical signs. This found reflex the risk to consumer by gastric cancer (26; 27; 28).

Some chemical compounds present in toxic plant could affect at human people by its ingestion not only direct so by food chain to consume products from intoxicate animals (1; 29). Preview at present work not exist reference reports by ptaquiloside levels in milking samples as contaminant or residual in these complex matrix.

Major associate between fern bracken infestation and the apparition esophagi and gastric cancer in persons who habit in these areas, first report by Evans et al., 1971 and in Costa Rica by Villalobos- Salazar, 1985, 1987, 1989 are associate with enough evidence by milk.

Some tumor were induce in rats and mice with oral administration of milk proceeding from cows with complement diets with fern bracken (6; 30; 31). Preview reports shown cattle had decrement in bone marrow activity with milk drinking from mother feed with bracken fern (32).

Milk of cattle grazing in pastures invaded by *Pteridium aquilinum* should be considered as a possible etiologic factor of gastric cancer in humans (3).Moreover, many chemical compounds that are present in harmful plants can affect human health not only for direct consumption of the plant but through the food chain by consuming milk and other products from animals intoxicated (13; 29).

The British Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) has declared ptaquilósido as a potential human carcinogen at all levels of intake (33).

Several studies have documented their genotoxic effects of mutagenic and clastogenic Pt acts as an alkylating agent of the DNA molecule (34; 35; 36; 37, 38).

Conclusion

Province Bolivar, Ecuador has a high risk by human health by the increasing in invasion for *Pteridium arachnoideum*. With high level of ptaquiloside in fronds and residual toxic elimination in cow milk.

Acknowledgement

Laboratorio Productos Naturales Vitales, Faculty of Chemistry, Universidad Central del Ecuador, Quito- Ecuador. Lic. Ximena Chiriboga.
Laboratory of Instrumental Analysis, Faculty of Chemistry, Universidad Central del Ecuador, Quito- Ecuador. Lic. Diego Lucero.

References

Rasmussen, LH., Lauren, D., Smith, B. and Hansen, HCB. (2008). Variation in ptaquiloside content In bracken (*Pteridium esculentum* (Forst. f) Cockayne) in New Zealand. N Z Vet J. 56(6): 304-309.

Kamon S and Hirayama T (1975) Epidemiology of cancer of the oesophagus in Mye, Nara and Wakayama prefecture with special reference to the role of brackenfern. *ProcJpn Cancer Assoc***34.**

Alonso-Amelot ME, Castillo U and De Jongh F (1993) On the passage of thebracken fern carcinogen, Ptaquiloside into bovine milk. *Le Lait***73**: 323–332.

Alonso-Amelot ME, Rodulfo-Baechler S and James-Espinosa R (1995) Comparative dynamics of Ptaquiloside and Pterosin B in the two varieties (*Caudatum* and *Arachnoideum*) of neotropical bracken fern (*Pteridium aquilinum*L. Kuhn).*BiochemSysyEcol***23**: 709–716.

Villalobos-Salazar J (1985) Carcinogenicidad del *Pteridium aquilinum* y alta incidencia del cáncer gástrico en Costa Rica. *RevCostCiencMed***6**: 131–139.

Evans IA, Jones RS and Mainwaring-Burton R (1972).Passage of bracken fern toxicity into milk. *Nature* **237**: 107.

Villalobos-Salazar J, Menenses A and Sales J (1990) Carcinogenic effects in mice of milk from cows fed on bracken fern, In Bracken Biology and Management, Thomson LA and Smith RT (eds) *AIAS Occasional Publication***40**: 247–251.

Wells AJ and McNally R (1989). Appraisal of the spatial association of bracken andcancer in England and Wales, presented at *Bracken: An Environmental Issue*, University of Wales, Aberystwyth.

Evans WC, Smith RT and Taylor JA (1986). The acute diseases caused by bracken in animals. In Bracken Ecology, Land Use and Control Technology, Smith RT and Taylor JA (eds), pp 139–146: Parthenon Publications: Carnforth, UK.

Villalobos-Salazar J, Mora J, Menenses A and Pashov B (1995) The carcinogenic effect of bracken spores, presented at *Bracken, an Environmental Issue*, University of Wales, Aberystwyth: 102–103.

Povey AC, Potter D and O'Connor PJ (1996) 32P-postlabelling analysis of DNA adducts formed in the upper gastrointestinal tissue of mice fed bracken extractor bracken spores. *Br J Cancer* **74**: 1342

Potter DM and Baird MS. 2000. Carcinogenic effects of ptaquiloside in bracken fern and related compounds. British Journal of Cancer (2000) **83**(7), 914–920.

Rasmussen, Lars Holm. (2003). PTAQUILOSIDE – AN ENVIRONMENTAL HAZARD? Occurrence and fate of a Bracken (*Pteridium* sp.) toxin in terrestrial environments. Thesis conducted under the Ph.D.-programme for Natural Sciences at The Royal Veterinary and Agricultural University.

Calderón Tobar, Ángela; Evangelina Marrero Faz; V. Murillo and V. Vega (2011). Reporte de casos de hematuria enzoótica bovina por ingestión de *Pteridium arachnoideum* en la región ganadera de San Miguel de Bolívar, provincia Bolívar, Ecuador. *Rev. Salud Anim. Vol. 33 No. 3: 197-202.*

Agnew, M.P. and Lauren, D.R. (1991) Determination of ptaquiloside in Bracken fern (*Pteridium esculentum*). Journal of Chromatography **538**, 462-468.

Quattrocchi OA, Abelaira S, Laba RF. Introducción al HPLC. Aplicación y Práctica. Buenos Aires: Artes Gráficas; 1992, p. 302 -28.

Attya Mohamed, Monica Nardi, Antonio Tagarelli and Giovanni Sindona (2012). A New Facile Synthesis of D4-Pterosin B and D4-Bromopterosin,Deuterated Analogues of Ptaquiloside. *Molecules 17*, 5795-5802. www.mdpi.com/journal/molecules.

Agnew, M.P. and Lauren, D.R. (1991) Determination of ptaquiloside in Bracken fern (*Pteridium esculentum*). Journal of Chromatography **538**, 462-468.

Santos, HM, Lodeiro, C. y CapeloMartínez, JL (2009) The Power of Ultrasound, in Ultrasound in Chemistry: Analytical Applications (ed J.-L. Capelo-Martínez), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim,Germany. http://onlinelibrary.wiley.com/book/10.1002/9783527623501

Villalobos-Salazar J, Mora J, Menenses A and Pashov B (1995).The carcinogenic effect of bracken spores, presented at *Bracken, an Environmental Issue*, University of Wales, Aberystwyth: 102–103.

Smith BL and Seawright AA. Bracken fern (Pteridium spp.) carcinogenicity and human health--a brief review. Nat Toxins.1995; 3(1): 1-5.

Rasmussen, LH., Jensen, LS and Hansen, HCB. (2003c). Distribution of the carcinogenic terpene ptaquilósido in bracken fronds, rhizomes (*Pteridium aquilinum*), and litter in Denmark. J Chem Ecol 29:771–778.

Somvanshi R, Lauren DR, Smith BL, et al. (2010). Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken. CURRENT SCIENCE, VOL. 91, NO. 11, 1547- 1552.

Saito, K., T. Nagao, M. Matoba, et al. (1989). Chemical assay of Ptaquiloside, the carcinogen of *Pteridium aquilinum*, and the distribution of related compounds in the Pteridaceae. Schery, R.W. 1954. Plants for man. London: Allen &Univin.

Alonso-Amelot ME, Castillo U, Smith BL and Lauren DR (1996) Bracken ptaquiloside in milk. *Nature***382**: 587.

Alonso-Amelot ME, Castillo UF, Avendaño M, Smith BL and Lauren DR. Milk as a vehicle for the transfer of ptaquiloside: a Bracken carcinogen. In: Taylor JA and Smith RT. (Eds.) Bracken Fern: Toxicity, Biology and Control. 2000. pp. 86-90.

Alonso-Amelot, ME., Avendaño, M. Possible association between gastric cancer and bracken fern in Venezuela: an epidemiologic study. Int J Cancer.2001; 91:252–259.

Alvarado DM. Evolución de algunos aspectos epidemiológicos y ecológicos del cáncer gástrico en Costa Rica. Rev. Costa Rric. Salud Pública. 2003;12:21.

DeLuca TH, Zewdie SA, Zackrisson O, Healey JR and Jones DL. Bracken fern (*Pteridium aquilinum* L. kuhn) promotes an open nitrogen cycle in heathland soils. Plant and Soil. 2012. [en línea].http://dx.doi.org/10.1007/s11104-012-1484-0.

Pamucku, A.M. and G.T. Bryan. 1979. Bracken fern, a natural urinary bladder and intestinal carcinogen. In: Miller, E.C. J.A. Miller, I. Hirono, T. Sugimura & S. Takayama (eds.). Naturally-Ocurring Carcinogens Mutagens and Modulators of Carcinogens. Scientific Societies Press, Tokyo, PP 89-99.

Villalobos, J.,A. Meneses and J. Salas. 1990. Carcinogenic effects in mice of milk from cows fed on bracken fern *Pteridium aquilinum*. In: J.A. Thomson and R.T. Smith (eds.) Bracken Biology and Management. AIAS Occasional Publication N2 40:247-251.

Evans, WC. 1986. The acute diseases caused by bracken in animals. In; R.T. Smith and JA. Taylor (eds). Bracken, Parthenon Publishing, Lancs, England. 121-132.

Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment, COT. (2008). Statement on the Risk to Consumers of Eating Foods Derived from Animals that Have Eaten Bracken, Food Standards Agency, London. pp. 20–21.

Almeida Santos, MF., Dorea, JG. and Luna, H.(2006). Bracken-fern extracts can be clastogenic or aneugenig depending on the tissus cell assay. Food ChemToxicol. 44(11):1845-1848.

Ferguson, LR. And Philpott, M. (2008). Nutrition and mutagenesis. Rev. Annu Rev Nutr. 28: 313-329.

Pereira, LO., Bicalho, Campos, PLM., de Sousa, TM., Bao, SN., de Fatima Menezes, ASM and Fonseca, MJ. (2009). DNA damage and apoptosis induced by *Pteridium aquilinum* aqueous extract in the oral cell lines HSG and OSCC-3. J Oral Pathol Med. 38(5):441-447.

Gil da Costa, RM., Bastos, MMSM., Oliveira, PA and Lopes, C. (2012). Bracken-associated human and animal health hazards: Chemical, biological and pathological evidence. Journal of Hazardous Materials. 203-204: 1-12.

Gil da Costa, RM., Coelho, P., Sousa, R., Bastos, MM., Porto, B., Teixeira, JP., Malheiro, I. and Lopes, C. (2012a). Multiple genotoxic activities of ptaquiloside in human lymphocytes: aneugenesis, clastogenesis and induction of sister chromatid exchange. Mutat Res. 747(1): 77-81.